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Chemical Composition of *Pinus brutia* Ten Essential Oil and Its *in vitro* Anti-Inflammatory Activity

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Authors' contributions

This work was carried out in collaboration among all authors. Author LK performed the experiments; wrote the paper; analyzed and interpreted the data. Author RN designed the experiments; analyzed and interpreted the data. All authors read and approved the final manuscript.

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ABSTRACT

This investigation aims to determine the chemical composition of *Pinus brutia* leaves essential oil and evaluate its anti-inflammatory property using Human Red Blood Cells (HRBC) membrane stabilization assay and Albumin denaturation assay. The chemical composition of essential oil (EO) obtained by hydro-distillation of leaves of *Pinus brutia* was investigated by GC-MS. The anti-inflammatory effect of EO was evaluated using Human Red Blood Cells (HRBC) membrane stabilization assay and Albumin denaturation assay. The main constituents of EO were α-Terpineol (66.16%), 3-Carene (4.90%), Carveol (4.55%) and cis-Verbenol (3.22%). The inhibion of hemolysis was observed at concentrations (2.5-12.5) µg/ml. Moreover, albumin denaturation test showed protection effect at concentrations (8-40) µg/ml. We concluded that, *Pinusbrutia* EO shows strong anti-inflammatory activity at different concentration when compared to standard drug of Diclofenac sodium. In addition, GC-MS analysis of *Pinus brutia* EO showed the presence of α-Terpineol as major compound in the oil. It reveals that this constituent is responsible to maximum protection of albumin denaturation and membrane stabilization assay. The future work will be determination of anti-inflammatory by *in vivo* models.

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1. INTRODUCTION

Essential oils, as defined by the European Pharmacopeia 7th edition, are "Odorant products, which have the complex composition, and obtained from plant raw extract, either extracted by steam of water, dry distillation or a suitable mechanical method without heating. Generally, a physical method is used for the separation of essential oil from the aqueous phase which has no significant change in its chemical composition" [1].

Essential oils found in many different plants, especially the aromatic and characterized in a number of families such as: Asteraceae, Lamiaceae, Lauraceae, Myrtaceae, Rutaceae, Cuppressaceae and Piperaceae [2]. However, parts of plants, which serve as the major source of essential oil can be different. Those include roots (valerian), peels (lemon), leaves (mint, pine), fruits (black pepper), barks (Cinnamon) and flowers (chamomile) [3].

Essential oils compounds can be divided into two main groups: terpene hydrocarbons (monoterpenes and sesquerpenes) and their oxygenated compounds such as: Phenols, Aldehydes, Ketones, Esters, Lactones and Ethers [4].

Many methods can be used to extract essential oils, which are dependent on botanical material used. Extraction techniques can be divided into two categories:

1. Classical methods (Steam distillation, Hydro-distillation and Solvent extraction).
2. Innovative methods Such as supercritical fluid extraction and Microwaves assisted extraction [5].

Essential oils have many applications in various industries, such as food products, drinks, perfumes, pharmaceuticals and cosmetic [6]. Therapeutic properties varies from plants to another, in Table 1 some examples of essential oils and their main active compounds as well as their therapeutic effects:

Pinusbrutia Ten. (Pinaceae) is known by several other names, Turkish pine, Calabrian pine (from a naturalized population of the pine in Calabria, Southern Italy, from where the pine was first botanically described), East Mediterranean pine and Brutia pine.Pinus trees are used in many industries; the turpentine which is obtained mainly from pine trees is used in medicine, pharmacy, food, cosmetics, paint and coatings [36].

Moreover, the resin obtained from *Pinus brutia* was used traditionally to treat Stomach ulcer, cough and it was also applied externally to heal wounds [37].

According to ethnobotanical study of Pinus species in Turkey, 130 traditional medicinal and ethnobotanical studies published up to 2011 which were dealt different areas of Turkey are examined and the usages of Pinus species are compiled from 54 of them. It has 269 records that are to be proof of the wide range of ethnobotanical usages of pines. It is stated in the literature that the most important medicinal usage of Pinus species is for respiratory system diseases and inflammatory diseases [37].

Nature-based medicines are having increased attention in the quest for novel pharmacophores that hold the prospect of enhanced therapy.

This interest follows the World Health Organization's 2008 ratification of The Beijing Declaration, which promotes the safe and effective use of traditional and alternative medicines and claims greater assimilation of these into national health care systems in order to improve therapy with less cost and side effects [38].

Therefore, this study aims to evaluate the anti-inflammatory activity and determine the active compounds which may be responsible for this activity.

In this study, the essential oil composition of the leaves of *P. brutia* collected from Syria were analyzed by GC/MS system, and its anti-inflammatory property was tested in-vitro using HRBC test and Albumin denaturation assy.

Table 1. Examples of essential oils and their main active compounds as well as their therapeutic effects

No	Essential oil	Main active compounds	Properties
	Chamomille essential oil	Bisabolol and chamazulene [7,8]	Anti-inflammatory, anti-allergic, decongestive (decongest the skin), anti-pruritic healing and antispasmodic [9]
	Dill essential oil	Carvone. [10]	Antispasmodic in gastrointestinal disorders [11]
	Garlic essential oil	Diallylle disulfide [12,13]	Maintains and protects the cardiovascular system, hypoglycemic, Regulates blood pressure vermifuge, antimicrobial, antiviral, anti-fungal and anti-parasitic, insecticidal and larvicidal, antioxidant [14]
	Clove essential oil	Eugenol and eugenyle acetate [15]	Antimicrobial, antiviral, antifungal, general stimulating, hypertensive aphrodisiac, carminative, anesthetic [16–19]
	Cinnamon essential oil	Cinnamaldehyde [20]	Antibacterial, antiviral, antifungal and parasiticide [21]
	Eucalyptus essential oil	1, 8-cineole [22]	Anticatarrhale, mucolytic and expectorant, antimicrobial, Antiviral [23–25]
	Peppermint essential oil	Menthol and menthone [26]	Tonic and stimulant, decongestant, anesthetic and analgesic antipruritic, refreshing, antimicrobial, anti-inflammatory, expectorant, mucolytic, emmenagogue [27–30]
	Lavender essential oil	Linalol and linalyle acetate [31,32]	Antispasmodic, relaxing, sedative, analgesic, anti-inflammatory and antimicrobial [33]
	Tea tree essential oil	Terpinene-1-ol-4 [34]	Antimicrobial, antiviral, antiasthenic, neurotonic, lymphatic, decongestant, radioprotective, antispasmodic [35]

2. MATERIAIS AND METHODS

2.1 Instrumentation and Apparatus

1	Electronic balance (Sartoruis AG, Germany)
2	Ultrapure TM water purification system (Lotun Co., Ltd., Taipei, Taiwan)
3	UV-1800 spectrophotometer (Shimadzu, Japan)
4	Water bath
5	Gas chromatography/mass spectrometry(Agilent, United States)

2.2 Materials and Reagents

Chemical materials: Sodium phosphate dibasic dehydrate (sigma Aldrich, Germany), Sodium phosphate monobasic dihydrate (Acros organics, United States), Distilled deionized water, Dimethyl sulfoxide- DMSO (sigma Aldrich, Germany), Sodium Chloride (HiMedia Laboratories, India) and Sodium Diclofenac (Amoli Organics Pvt. India).

Plant source: The leaves of *Pinus brutia* were collected in March from classified trees growing in the campus of Aleppo University. The leaves were dried in the shade in a well-ventilated place, then stored in airtight containers.

2.3 Methods

2.3.1 Isolation of the essential oil

Air-dried leaves (100 g) were subjected to hydro distillation using a Clevenger-type apparatus for 3h.

2.3.2 Gas chromatography and mass spectro metry (GC-MS) analysis

The oil was analyzed by GC-MS, using a Hewlett Packard system. HP-Agilent 5975 N GC-MS system with 7890 GC in Aleppo University.

Method was done according to Bagci et al with some modification: [39]

DB-5 HT column (30 m × 0.32 mm i.d., film tickness 0.1µm) was used with helium as the carrier gas. Injector volume was 10µl, split ratio was 1:10, and front inlet temperature was 290 ºC. The GC oven temperature was kept at 60ºC for 0 min and programmed to 280ºC at a rate of 5ºC/min and then kept constant at 280ºC for 1 min.

Component identification was carried out using spectrometric electronic libraries (WILEY, NIST).

2.3.3 Evaluation of anti-inflammatory activity

The anti-inflammatory activity of essential oil of *Pinus brutia* leaves was evaluated by human red blood cell (HRBC) membrane stabilization and albumin denaturation assay.

2.3.3.1 HRBC membrane stabilization assay

The effects of the essential oil on hemolysis of HRBC induced by heat was evaluated using the method of Shinde et al. with some modifications [40].

2.3.3.1.1 Preparation of erythrocyte suspension

Fresh whole blood (3 ml) was collected from healthy volunteers, who were nonsmokers, did not take alcoholic drinks, and did not use any chemical medicine for one week; the samples were put into heparinized tubes then centrifuged at 3000 rpm for 10 min. A volume of normal saline equivalent to that of the supernatant was used to dissolve the red blood cells. The volume of the dissolved red blood cells obtained was measured and reconstituted as a 40% suspension with isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4). The buffer solution contained 0.2g of NaH2PO4, 1.15 g of Na2HPO4 and 9 g of NaCl in 1liter of distilled water.

2.3.3.1.2 Assay of membrane stabilization by heat induced hemolysis

Essential oil was dissolved by Dimethyl sulfoxide (DMSO) 10% to obtain concentration (5-10-15-20-25 µg/ml).

Two groups of centrifuge tubes were prepared in such a way that each tube contained 5 mL of essential oil, 4.85 mL of isotonic buffer solution and 0.15mL of HRBC suspension 40%. One of the group was incubated in a water bath at 54ºC for 20 minutes. The other group was placed in the refrigerator. The negative control was prepared by putting saline instead of essential oil and Sodium Diclofenac was used as a standard drug.

Afterwards, all tubes were centrifuged at 3500 rpm for 7 min and the absorbance of the supernatants was taken at 560nm. The experiment was performed in triplicate. Percentage of membrane stabilization activity was calculated by the formula mentioned below:

$$\text{Protection \%} = 1 - \left(\frac{OD2 - OD1}{OD3 - OD1}\right) * 100$$

Where:

OD1= absorbance of test sample unheated
OD2 = absorbance of test sample heated
OD3=absorbance of control sample heated.

2.3.3.2 Evaluation of essential oil effect on albumin denaturation

The effects of the *Pinus brutia* essential oil on Albumin denaturation was evaluated using the method of Chatterjee et al. with some modifications [41].

The reaction mixture contains 2.8 ml of isotonic phosphate buffer (10 mM sodium phosphate buffer, pH 7.4), 0.2 ml of egg albumin (from hen eggs) and 2 ml of different concentrations from Essential oil (20-40-50-60-100) µg/ml. Distilled water was used as a negative control and Sodium Diclofenac was used as a positive control.

The reaction mixture was incubated at 37ºC for 20 minutes then incubated at 70ºC for 5 minutes. After cooling, the absorbance was measured at 660nm, the percentage of protection of protein denaturation was calculated as in the following equation: [42]

Protection% = 1- ((studied sample absorbance) / (negative control absorbance)) * 100.

2.3.4 Statistical study

All experiments were performed triplicates; results were expressed as mean values ± standard deviation (SD). Statistical analysis was carried out using the Statistical Package for the Social Science SPSS Version 22. Results were statistically analyzed by one-way ANOVA. The p-value<0.05 was statistically significant when compared with control.

3. RESULTS AND DISCUSSION

3.1 GC-MS Analysis

Yield of oil was 0.38%, Gas chromatogram of the essential oil from dried leaves is shown in Fig. 1. The identified constituents of the essential oils are listed in Table 2.

In the essential oil of *Pinus brutia* leaves, the main components were identified and constituting 82.93%.

The major components of *Pinus brutia* leaves were α-Terpineol (66.16%), 3-Carene (4.90%), Carveol (4.55%) and cis-Verbenol (3.22%).

Experimental results from our study, concerning the composition of essential oils, are in accordance with previously published data. The composition of the essential oils isolated from the flowers and cones of *Pinus brutia* grown in Lebanon were investigated and the main components were monoterpenes and oxygenated monoterpenes such as α-Pinene, β-Pinene and Terpinen-4-ol [43].

On the other hand, according to a study which was carried out in Tunisia, the essential oil of *Pinus brutia* leaves was characterized with presence of further component such as: Thujene and phellandrene [44].

3.2 Assay of Membrane Stabilization by Heat Induced Hemolysis

Essential oil of *Pinus brutia* Leaves showed efficiency in membrane stabilization by heat induced hemolysis as shown in Table 3. Whereas the oil showed the highest efficiency which reached 56.71% at concentration 10µg/ml.

Lysosomes are one of the factors that may contribute to tissue damage during the inflammatory process, by oxidizing cell membrane lipids.

In 1979, Studies also showed that lysosomes inhibit the steroid anti-inflammatory receptor Hsp90 by changing it to a smaller form, preventing its joining with the steroidal anti-inflammatory drug, which increases the inflammatory state [45].

Therefore, the stabilization of the lysosome membrane contributes in preventing the mediators release such as proteases and

reducing the inflammatory response. The erythrocyte membrane is similar to the lysosome membrane. Thus, the anti-hemolytic effect of plant essential oil can be taken as evidence of the anti-inflammatory effectiveness [42].

3.3 Evaluation of Essential Oil Effect on Albumin Denaturation

Essential oil showed efficiency in albumin denaturation assay as shown in Table 4. The oil showed the highest efficiency which reached 55.38% at concentration 40 µg/ml, while sodium Diclofenac did not give a noticeable efficacy at the concentration of 40 µg/ml.

Researchers found that denaturation of protein is one of the causes of rheumatoid arthritis. Production of auto-antigen in certain arthritic diseases may be due to denaturation of protein [46].

The anti-inflammatory property of essential oils may be explained by considering that essential oil are able to scavenge some free radicals, which play major role in inflammatory response [47] and this is in accordance with traditional use where Pinus species have been used against rheumatic pain and inflammatory cases [48].

Furthermore, in a study carried out to evaluate the anti-inflammatory property of alpha-terpineol, revealed that alpha-terpineol had an inhibiting effect on IL-6 formation. This anti-inflammatory effect of alpha-terpineol on IL-6 formation was verified by quantitative real-time reverse transcription Polymerase Chain Reaction experiments in which alpha-terpineol inhibited the gene expression of the IL-6 receptor [49].

Table 2. Percentage composition of essential oil from *Pinus brutia* leaves

No	Compound	Percentage%
	α-Terpineol	66.16
	Carveol	4.55
	Berbenol	1.05
	Aromadendrene	2.10
	cis-Verbenol	3.22
	3-Carene	4.90
	Cinen	0.95

Table 3. Effect of *Pinus brutia* essential oil on heat induced hemolysis of HRBCs

Treatment	Concentration µg/ml	Protection% Mean±SD
Pinus brutia Essential oil	2.5	48.04±8.36
	5	49.03±1.82
	7.5	55.80±1.39
	10	56.71±2.98
	12.5	55.71±2.73
Sodium Diclofenac	100	89.21±2.54

Table 4. Effect of *Pinusbriutia* essential oil on albumin denaturation

Treatment	Concentration µg/ml	Protection% Mean±SD
Pinus brutia Essential oil	8	15.25±3.05
	16	22.7±6.11
	20	24.17±1.27
	24	31.51±2.06
	40	55.38±4.16
Sodium Diclofenac	40	-
	120	11.02±2.70
	160	22.80±1.30

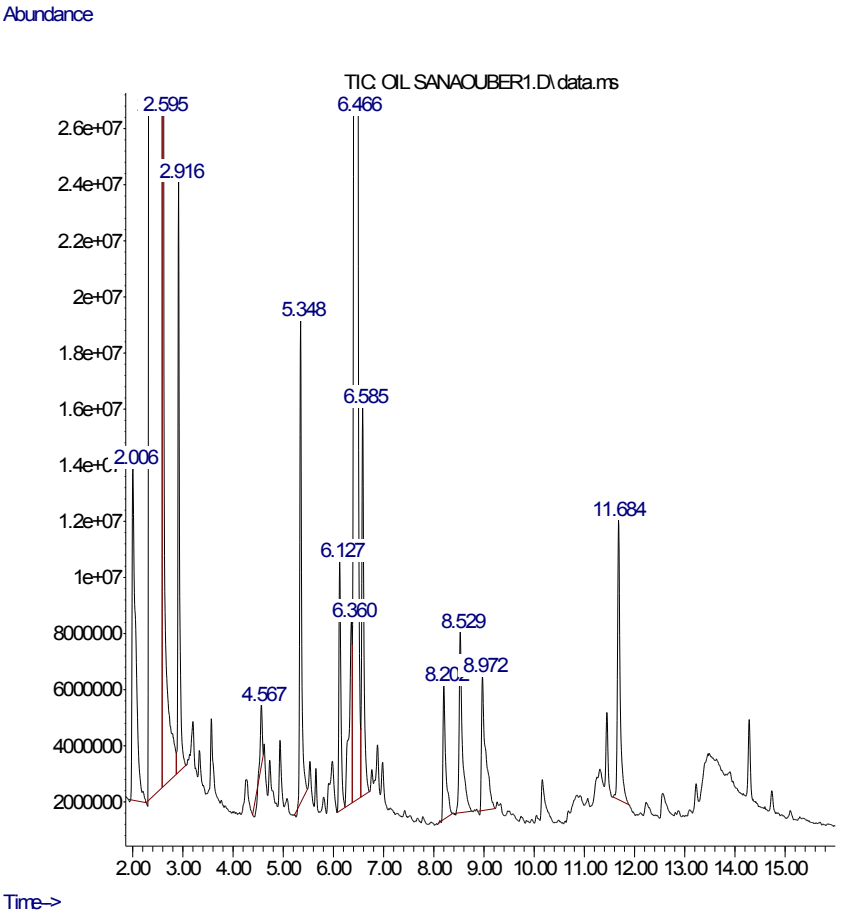

Fig. 1. Gas chromatogram of the essential oil from dried leaves of *Pinus brutia*

4. CONCLUSION

The GC/MS analysis proves that the major compound of *Pinus brutia* leaves essential oil was α-Terpineol, which may attribute to the anti-inflammatory activity of EO. The in-vitro anti-inflammatory tests which were carried out in this study indicate strong anti-inflammatory effect of *Pinus brutia* EO: further in vivo studies are needed to ensure this activity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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